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Biogeography, population genetics and mating systems of natural *Nasonia* populations

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Chapter 2

Genetic structure of natural *Nasonia vitripennis* populations: validating assumptions of sex ratio theory

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Abstract

The parasitic wasp *Nasonia vitripennis* has been used extensively in sex allocation research. Although laboratory experiments have largely confirmed predictions of Local Mate Competition (LMC) theory, the underlying assumptions of LMC models have hardly been explored in nature. We genotyped over 3500 individuals from two distant locations (in the Netherlands and Germany) at four polymorphic microsatellite loci to validate key assumptions of LMC theory, in terms of both the original models and more recent extensions to them. We estimated the number of females contributing eggs to patches of hosts and the clutch sizes as well as sex ratios produced by individual foundresses. In addition we evaluated the level of inbreeding and population differentiation. Foundress numbers ranged from 1 to 7 (average 3.0 ± 0.46 SE). Foundresses were randomly distributed across the patches and across hosts within patches, with few parasitizing more than one patch. Of the hosts, 40% were parasitized by more than one foundress. Clutch sizes of individual foundresses (average 9.99 ± 0.51 SE) varied considerably between hosts. The time period during which offspring continued to emerge from a patch or host correlated strongly with foundress number, indicating that sequential rather than simultaneous parasitism is the more common. Genetic differentiation at the regional level between Germany and the Netherlands, as estimated by Slatkin's private allele method (0.11) and Hedrick's corrected G'_{LT} (0.23), indicates significant substructuring between regions. The level of population inbreeding for the two localities ($F_{IL} = 0.168$) fitted the expectation based on the average foundress number per patch.

Introduction

Local mate competition (LMC) theory (Hamilton 1967) is the basis for a large amount of research into adaptive sex ratio adjustment (Werren 1983; Herre 1985; Orzack 1986; King & Skinner 1991; Hardy 1994; Godfray & Werren 1996; Antolin 1999; Courteau & Lessard 2000; West *et al.* 2000; Shuker *et al.* 2004b; 2005). It assumes that a female has control over the sex ratio of her offspring and can maximize her fitness by reducing the competition between her sons. This is an evolutionary stable strategy if males are not the dispersing sex and if mating only takes place at the natal patch (Hamilton 1967). In such a mating system all males are competing to mate with the females that are available at the patch. If the patch population consists of only a single family, the males are brothers and it is beneficial for the foundress female to shift the offspring sex ratio strongly towards daughters to reduce competition among her sons. With increasing foundress number, competition between unrelated males increases and therefore selection favors females that produce more males to increase the chance that their sons mate with daughters of other females as well. This leads to a less female-biased sex ratio. The resulting prediction is that the offspring sex ratio in a patch is a function of the number of females ovipositing on that patch (Hamilton 1967).

A central assumption of LMC theory is that the population is highly subdivided in terms of mating. In the case of parasites this is thought to be due to the patchy distribution of hosts. Hamilton (1967) assumes that clutch sizes are equal and that there is random mating among the offspring of one patch. A patch could be for example all the fly pupae on a carcass or in a bird nest. The resulting population inbreeding F_{IT} follows $F_{IT} = 1 / (4n-3)$ with n being the mean number of foundresses per patch (Hamilton 1979).

Hamilton's LMC model has been further extended by several authors in various ways. The concept that females can have different clutch sizes and sex ratios has been incorporated by Werren (1980). Inbreeding has also been considered in several ways (Herre 1985; Frank 1985b). Nunney and Luck (1988) modeled the combined effects of male dispersal, inbreeding and asynchronous parasitism on sex allocation, whilst Courteau and Lessard (2000) in turn developed several different scenarios of dispersal, i.e. before or after

mating and dispersal probability for haploid, diploid and haplo-diploid organisms. Shuker *et al.* (2005) recently extended Nunney and Luck's (1988) model of asynchronous parasitism by considering two foundresses parasitizing hosts on a patch sequentially but allowing females to use either the same or different hosts. In species such as the parasitic wasp *Nasonia vitripennis* asynchronous parasitism on a single host is thought to have little effect on the timing of emergence, as *N. vitripennis* larvae speed up their development to achieve a synchronous emergence of all individuals from a host (Werren 1980). In contrast asynchronous parasitism of several hosts in a patch leads to asynchronous emergence of the offspring. As such, males of an early foundress have a chance to mate with females of a later foundress, whose sons do not have access to the daughters of the early foundress. Such asymmetric LMC leads to a shift of the optimal sex ratio towards more males for the second foundress (Shuker *et al.* 2005). Like most models, Shuker *et al.*'s (2005) model has been confirmed under laboratory conditions (see also Shuker *et al.* 2006b), but few field studies have been performed to test these models.

The parasitoid wasp *Nasonia vitripennis* has been widely used for laboratory experiments regarding sex ratio adjustment and behavioral genetics (Werren 1984; Drapeau & Werren 1999; Beukeboom & van den Assem 2001; van den Assem & Beukeboom 2004; Shuker *et al.* 2005; 2006b). Laboratory experiments and two field studies (Werren 1983; Molbo & Parker 1996) have shown that *N. vitripennis* modulates the sex ratio of its offspring largely consistent with LMC theory. As Molbo and Parker (1996) used allozymes, which have a rather low variability, it is possible that they underestimated foundress number. In addition the level of superparasitism might also have been underestimated, as they themselves acknowledged. Werren (1983) on the other hand used the offspring number per patch as an indirect measure of the foundress number and found a strong positive correlation between patch offspring number and sex ratio, leveling off at 50% males.

Other genetic studies on parasitoid Hymenoptera have considered the level of the population rather than the level of individual patches, and have produced varying results on the population substructuring and the level of inbreeding. De Leon and Jones (2005) found for *Gonatocerus ashmeadi* a pronounced genetic structure between samples from the American East- and West coast ($G_{ST} =$

0.38), while Kankare *et al.* (2005) found differing results for *Cotesia melitaearum* and *Hyposoter horticola*. F_{ST} for *C. melitaearum* was much higher than for *H. horticola* (0.378 vs. 0.063), and both species showed significant isolation by distance. These differences between parasites of the same host species reflect their differences in mobility (Kankare *et al.* 2005). In a study on *Trichogramma pretiosum* (Antolin 1999), a rather high degree of population inbreeding ($F_{IT} = 0.246$) was found but no significant differentiation between three subpopulations within California. These different findings regarding the population structure of various parasitoid wasps do not allow any generalizations, and do not specifically test assumptions of LMC. In this study we use four polymorphic microsatellites to estimate the level of inbreeding, foundress numbers, timing of parasitism and individual sex allocation in two field populations of *N. vitripennis* in Europe in order to test how well natural populations represent the idealized conditions assumed in models of LMC.

Material and Methods

Sampling

Nasonia vitripennis is a gregarious pupal parasitoid of a wide range of cyclorrhaphous flies. Like all Hymenoptera, *N. vitripennis* has a haplodiploid reproduction mode: fertilized eggs develop into diploid females, unfertilized eggs into haploid males. In *N. vitripennis* females usually mate at their place of birth and disperse after mating. Males have reduced wings and cannot fly (Whiting 1967).

Fly host pupae were collected from bird nests obtained from 95 nest boxes in a 1.4 km x 2.5 km field site in the Hoge Veluwe National Park (The Netherlands) (referred to as HV) and from baits placed in all HV nest boxes. A second plot consisted of 28 nest boxes along a straight ~600 m long road near Schlüchtern (Hessen, Germany) (referred to as Schl), where only baits were used. The collected host pupae were incubated individually at room temperature (~20°C) and the emerging wasps, after being identified as *N. vitripennis*, were counted, sexed and stored directly in 90% ethanol for molecular analysis. For the HV-samples we kept record of the first and last day of emergence for every host pupa. Unfortunately we could not record the data per individual wasp. For baiting (in Schl), 25 laboratory hosts (*Calliphora vicina*) were placed in a mesh

bag and left inside the nest box for approximately one week to allow parasitism. As the nest boxes are cleaned out every year and we did not find any host pupae that showed signs of emergence, we assume that our sample represents all offspring that emerged from these nest boxes.

Parentage analysis

DNA isolation followed a standard high salt-chloroform protocol (Maniatis *et al.* 1982). For genotyping we used four polymorphic microsatellites (dinucleotide repeats) (Table 2.1). Nv-22 and Nv-23 have originally been developed by Pietsch *et al.* (2004) but the primers have been redesigned in our laboratory. Primer sets for the other two microsatellites have been developed in our laboratory using the technique described by Rütten *et al.* (2001). The length of the amplified fragments was determined on an ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

The genotypes of the females (here called foundresses) that oviposited on each host were determined from the genotypic data of the offspring following these simple rules: (1) A female can maximally provide two alleles per locus. (2) The father can only provide one allele per locus (being haploid) that is shared by all full sisters. (3) Sons can only have an allele from their mother, as they develop from unfertilized eggs. If several foundress genotypes were possible based on the microsatellite profile, we always preferred the solution with the lowest number of foundresses. We allowed the foundresses to be multiply mated in our paternity analysis. This foundress assignment has been done independently by three of the authors (BKG, TK and MNB-C) to validate the assignment process. It yielded data on the number of foundresses per nest box and per host, as well as on the individual clutch sizes and sex ratio of every foundress.

Table 2.1: Chromosomal location, primer sequences, number of alleles, Nei's overall gene diversity (H_t) (Nei 1987) and annealing temperatures of four microsatellites used.

Primer	Chromo- some*	Sequence	Allele no.	H_t	Ann. temp.	GenBank accession no.
Nv-22	I	5' GAC TGC GTA CCA CTC CAA AAA TA 3' 5' AAG ACC AGC TAG GGA AGA GGA TA 3'	16	0.90	58°C	AY262041
Nv-23	II	5' ATA CTC AAG CAA GCC ACA GCA TA 3' 5' GCG TAC CAA TCC ACA GAA AAT AG 3'	13	0.39	58°C	AY262044
Nv-41	IV	5' GTC AGA CGT GGG CTT TGT C 3' 5' TTA TGC GCC ACA CAC ACC 3'	11	0.85	52°C	EU155141
Nv-46	V	5' TTA CGT CAA GGT ATA GCT GC 3' 5' GAA TAA GTG GCT GAA AGT TTC C 3'	27	0.87	58°C	EU155142

* chromosome designation according to Rütten *et al.* (2004)

Population structure analysis

Some sex allocation models use the population inbreeding coefficient F_{IT} as a measure of relatedness to estimate the optimal strategy for a foundress (e.g. Frank 1985b and see citations in introduction), assuming that females (and patches) have equal productivity. Furthermore, information about the population genetic structure allows estimations about gene flow and migration rates among populations. For this analysis we divided the samples into the two geographic regions (HV and Schl) which contain several nest boxes, each of which can be considered as a patch (in the LMC terminology) or a subpopulation (in the F -statistical sense). As the individuals emerging from one nest are the members of only a few families (Molbo & Parker 1996), the relatedness among these is very high. We therefore decided to use each foundress genotype once, rather than use the genotypes of all the offspring. In this way the sample size was reduced considerably, but we avoided multiple non-independent samples. The most common method for determining population differentiation and inbreeding involves F -statistics, which were originally designed for diploid organisms (Wright 1931; Weir & Cockerham 1984; Slatkin 1987; Cockerham & Weir 1993). As we only use the diploid females in our analysis, we can apply F -statistics. However, in their review on population genetics of X-linked genes and haplodiploids, Hedrick and Parker (1997) find that a major effect of

haplodiploid inheritance is a reduced effective population size compared to diploids. Hence, care should be taken in comparing quantitative results with data of diploid organisms.

Hedrick (1999) cautioned against the use of conventional F-statistics on microsatellite data, as the high mutation rate and the high number of alleles of such markers can lead to a severe underestimation of the genetic differentiation. New mutations in separated populations can produce identical alleles that are not identical by descent and therefore mask the differentiation (Nauta & Weissing 1996). He recommended the use of Slatkin's private allele method (Slatkin 1985; Barton & Slatkin 1985). Later Hedrick (2005) developed a standardized measure of F_{ST} , called G'_{ST} which is standardized for the maximal value that G_{ST} (a multi allelic version of F_{ST}) can reach, given a certain genetic diversity in a population. Here we apply all three methods and compare the results.

In the following we will use the F-statistical terminology as used by Hartl and Clark (1997), with the subpopulation (index S) being the individual nest box, or patch in the LMC sense, and the sampling areas (Schl or HV) being localities (index L). The total population (index T) represents the pooled data set of both localities. A classical F-statistical analysis within regions was not possible, as 5 out of the 18 nest boxes were parasitized by one female only (leading to very localised mating prior to female dispersal). As a substitute for F_{ST} we used Rousset's distance a (Rousset 2000) between pairs of individuals within a region, within and between patches to test for isolation by distance. The expectation is a linear positive correlation between genetic distance and the logarithmic geographic distance (Rousset 1997).

Population statistics were calculated using FSTAT 2.9.3 (Goudet 2001), GENEPOP (<http://genepop.curtin.edu.au/>) (Raymond & Rousset 1995), and SPAGeDi 1.2 (Hardy & Vekemans 2002). Statistical tests were performed with SPSS 13.0 or R 2.4.1 (R Development Core Team 2006). All mean values are given as arithmetic mean \pm SE unless indicated differently.

Table 2.2: Number of foundresses estimated, total number of hosts, and total number of parasitized hosts at 2 field sites. HV = Hoge Veluwe National Park (The Netherlands); Schl = Schlüchtern (Hessen, Germany);

Nest box no.	No. of foundresses	Total hosts	Hosts used
HV 8	1	15	1
HV 13	5	27	27
HV 220	5	NA	9
HV 267	7	16	16
HV 288	1	25	11
HV 306	1	6	1
HV 323	2	8	6
HV 330	5	82	79
HV 344	1	43	4
HV 365	1	35	1
Schl 11	4 (a)	25	15
Schl 13	2(b)	25	3
Schl 16	2	25	4
Schl 20	2(c)	25	25
Schl 21	7(a, d)	25	9
Schl 22	4(b, c, d)	25	14
Schl 23	2	25	1
Schl 28	3	25	15
Total	49 (6 double visits)	466	241

a = 3 foundresses found in nest box Schl 11 that also parasitized nest box Schl 21

b = foundress that parasitized nest box Schl 13 and Schl 22

c = foundress that parasitized nest box Schl 20 and Schl 22

d = foundress that parasitized nest box Schl 21 and Schl 22

NA = the number of total hosts in these nest boxes was not recorded; for the total number of hosts the number of hosts parasitized (9) was assumed

Results

Foundress numbers and pattern of parasitism

From the 95 nests that were inspected at the Hoge Veluwe, 15 (16%) contained fly pupae of which 9 (9.5% of total) yielded *Nasonia vitripennis* emerging from at least one host. The baits in the HV nest boxes only yielded *Nasonia* in one case (HV 288). From the 28 baited nest boxes in Schlüchtern, 8 (29%) yielded *N. vitripennis*. The total number of natural hosts found per nest box ranged from 6 to 82. The number of parasitized hosts per nest box ranged from 1 to 79 (Table 2.2).

We genotyped a total of 3550 individuals emerging from 9 natural nests (HV) and 9 baits (8 Schl and 1 HV) (the complete data can be found as Supplementary Data online). We could identify a total of 49 foundresses (arithmetic mean per patch: overall 3.0 ± 0.46 , in HV 2.9 ± 0.74 , in Schl 3.1 ± 0.55 ; harmonic means: overall 1.9, HV 1.6, Schl. 2.4, Figure 2.1A). Assuming that the allele frequencies measured in our sample represent the genetic makeup of the whole population (HV and Schl combined), the chance that two unrelated individuals share the same allele is equal to the frequency of the particular allele in the population. Due to the high allelic variation of our markers, the chance of encountering two or respectively three females that have identical genotype in all four markers is < 0.001 . The number of offspring per foundress per host varied between 1 and 39 (mean = 9.99 ± 0.41), while the number of hosts being parasitized by a single foundress varied between 1 and 27 (mean = 7.17 ± 1.02). The total number of offspring per foundress across all hosts varied between 1 and 346 (mean = 72.5 ± 9.46). The total number of offspring per host varied between 1 and 55 (mean = 14.77 ± 0.64). The observed level of superparasitism is high. In 39.5% (N = 241) of all hosts we found evidence for more than one foundress and in 5.5% more than two foundresses (Figure 2.1). In Schlüchtern we found six foundresses parasitizing hosts in two nest boxes each (three on S11 and S21, one on S13 and S22, and one on S20 and S22 Table 2.1). We found no significant difference in the distribution of foundresses across patches or hosts between the natural nests (HV samples) and baits (SCHL samples) (Kolmogorov-Smirnov test: patch-level D = 0.29 n.s., host-level D = 0.5 n.s.).

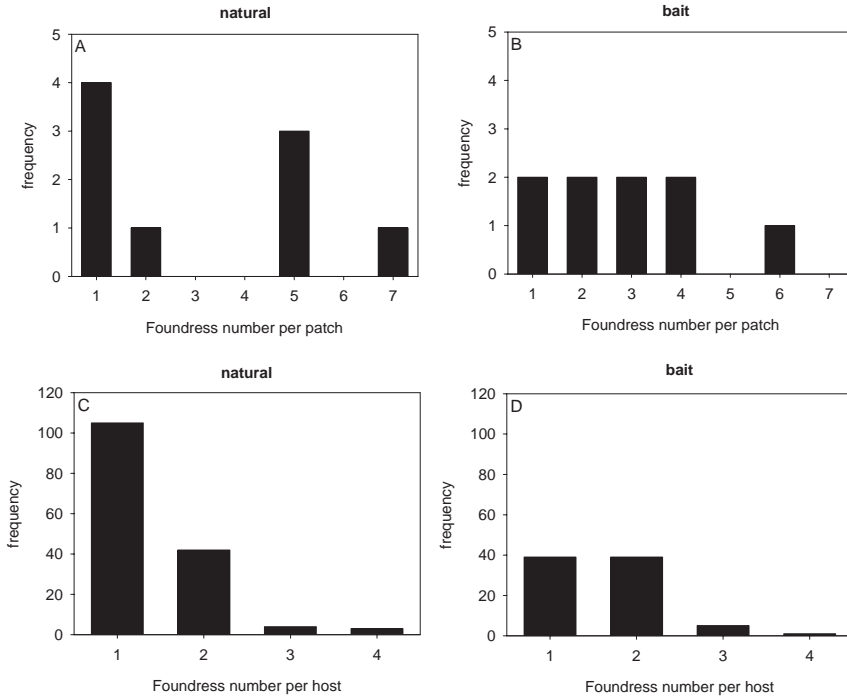


Figure 2.1: Frequency distribution of the number of foundresses per patch (A, B) and host (C, D) under natural conditions (A, C) and baits (B, D).

There was no evidence for female preference for or against patches or hosts used by other females. We found no significant deviation from a random distribution of the foundresses across used patches (dispersion test for a Poisson distribution following Grafen and Hails (2002), $\chi^2 = 69.03$, $df = 237$, $p = 1$) or across the hosts within a patch (χ^2 -test against Binomial distribution using pooled data of all patches and combining the low represented classes, $\chi^2 = 12$, $df = 9$, $p = 0.213$). The total number of hosts present in a patch and the number of foundresses parasitizing also showed no significant correlation (adj. $R^2 = -0.024$, $F_{1,17} = 0.582$, $p = 0.456$). Although there is a large variation in the clutch

sizes per foundress, there was no significant correlation between foundress number per host and the clutch size per foundress (adj. $R^2 = -0.003$, $F_{1,342} = 0.07$, $p = 0.798$). The mean coefficient of variation within clutch sizes of a particular host is 0.69 ± 0.04 , and therefore not negligible.

Although the data were not specifically collected to test for synchrony of parasitism, we can obtain some information from our data. The time window in which wasps emerged from a single host ranges from 1 to 10 days and for all hosts of a patch from 1 to 19 days (Figure 2.2). There is a strong positive relationship between foundress number and emergence window of a patch (adj. $R^2 = 0.7161$, $F_{1,7} = 21.18$, $\beta = 2.71 \pm 0.59$, $p = 0.0024$; Figure 2.2A) and of a host (adj. $R^2 = 0.093$, $F_{1,139} = 15.4$, $\beta = 0.95 \pm 0.24$, $p < 0.001$; Figure 2.2B).

Our data strongly suggest multiple mating in two cases (4%, $N = 49$). Among the HV-foundresses we found one female that was doubly mated (HV 267, foundress #14) and one that was mated three times (HV 330, foundress #26). An alternative explanation would be a genotyping error, but the decision on additional mates is supported by more than one marker, and doubtful individuals have been genotyped twice, which makes genotyping errors unlikely. The high level of inbreeding in the population (see data below) however, increases the relatedness of individuals in the populations and therefore the chance of highly related individuals parasitizing the same patch, which could then lead to the impression of multiple mating.

One nest produced only male offspring (HV 8), which can most easily be explained by a single unmated foundress. We excluded this progeny from further sex ratio analysis. Without this nest the sex ratio (proportion male) of the emerged offspring varied between 0.05 and 0.56 across the nests. The nest and host sex ratios as a function of foundress number roughly fit the theoretical predictions of basic LMC models (Figure 2.3).

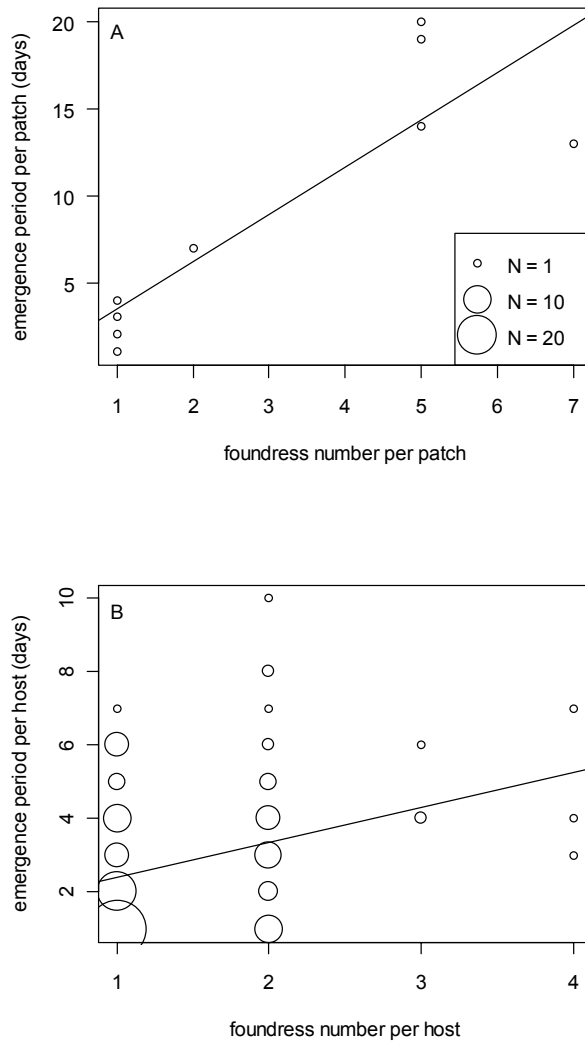


Figure 2.2: Emergence window per patch (A) and per host (B) in days as a function of the foundress number per patch (A) and per host (B). The circle surface is proportional to the sample size. The regression lines are highly significant (see text for details).

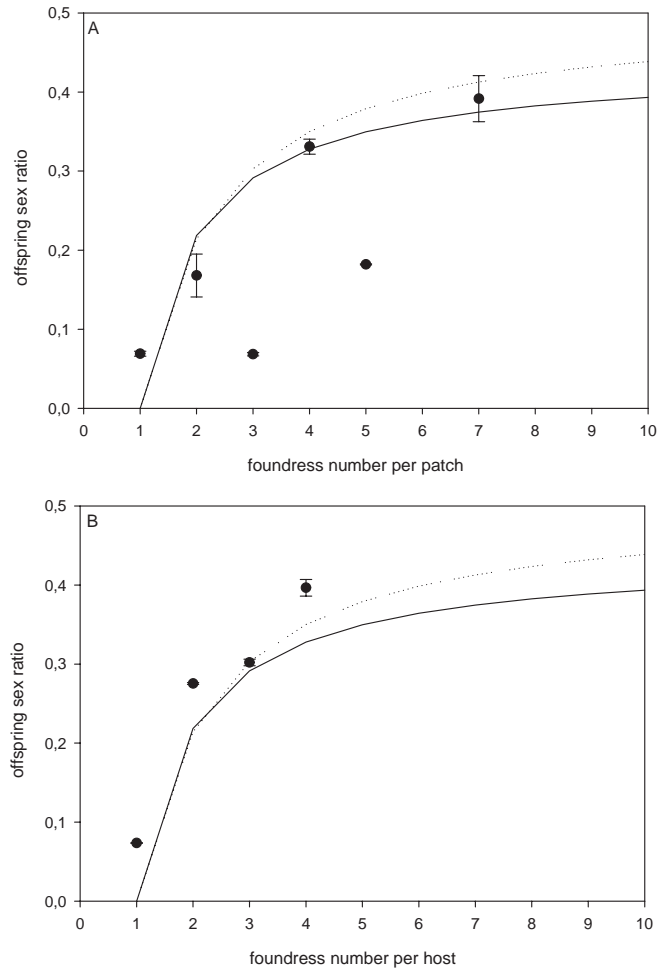


Figure 2.3: Sex ratio (proportion of males) \pm SE as a function of foundress number per nestbox (all hosts pooled) (A) and per host (B), compared to the expectation of Hamilton (1979) (dotted line) and Frank (1985) assuming $F_{IL} = 0.168$ (solid line).

Population genetic analysis

Due to the fact that *N. vitripennis* is mating in the natal patch, and the small size (~3 mm) of the dispersing females, the population of this parasitoid is expected to be highly subdivided. Random mating within the regions (Schl and HV) and the total population can be tested indirectly by comparing measured and expected heterozygosity among the foundress females. The mean $F_{IT} = 0.168 \pm 0.016$ indicates a heterozygote deficiency and therefore non-random mating (inbreeding) within the regions; $F_{IT} = 0.197 \pm 0.014$ shows more or less the same for the whole population (HV and Schl pooled) (Hartl & Clark 1997). The differentiation index $F_{LT} = 0.035 \pm 0.011$ indicates a low differentiation among the regions in general (averaging over loci). Slatkin's private allele method results in $Nm = 2.62$ which corresponds to $F_{LT} = 0.11$ (following $F_{RT} = 1/(1+3Nm)$), a threefold higher value. Hedrick's standardized $G'_{LT} = 0.23$ is even higher (Hedrick 2005).

We found no positive correlation between geographic (ln (geographic distance)) and genetic distance (Rousset's a) within a locality (Mantel's test: Schl $r^2 = 0.0047$, n.s.; HV $r^2 = 0.0043$, n.s., Figure 2.4). The mean genetic distance between foundress females of one patch was not different from the mean genetic distance of foundress females from different patches within one region (HV: within patches 0.19 ± 0.04 , between patches 0.21 ± 0.02 , 2-sided t-test: $t = -0.3833$, $df = 49.825$, n.s.; Schl: within patches 0.16 ± 0.07 , between patches 0.14 ± 0.02 , 2-sided t-test: $t = 0.1928$, $df = 17.09$, n.s.).

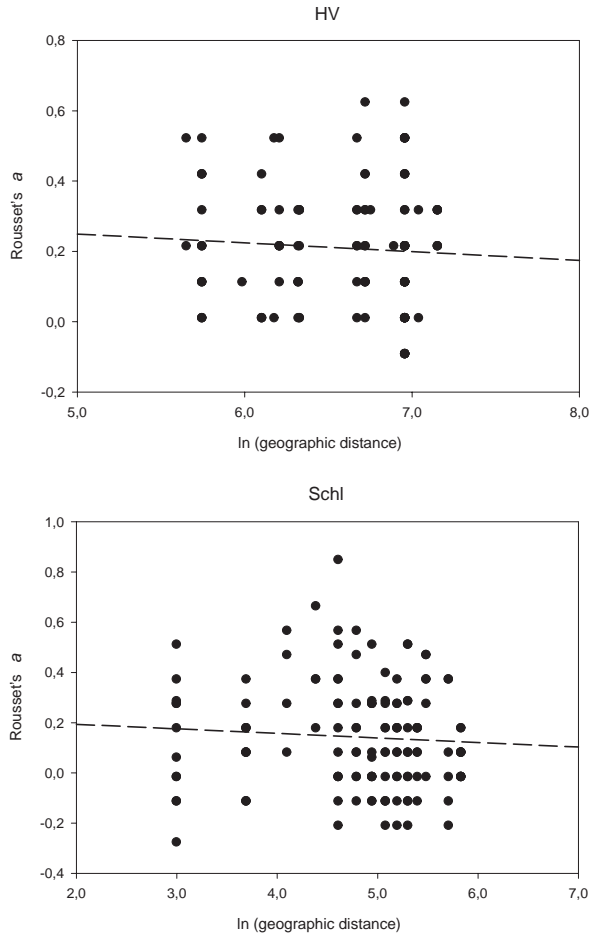


Figure 2.4: Genetic differentiation in *N. vitripennis*. Shown is pairwise genetic differentiation in the form of Rousset's a (Rousset 2000) against logarithmic geographic distance. The upper graph shows the HV data (R^2 of regression line 0.0043), the lower graph Schl (R^2 of regression line 0.0047). All pairs of estimated foundresses from different nest boxes are shown, as well as the regression lines.

Discussion

Herre (1985) found that species of fig wasp that are more likely to encounter a conspecific on a patch are more likely to shift sex ratios as predicted by LMC. *N. vitripennis* is known to have a very strong response to LMC in the laboratory. Here we have shown that foundress numbers vary across hosts and patches in the wild, with a high superparasitism rate of 40% at the host level and 63% at the patch level. Therefore we can conclude that conditions favouring facultative sex allocation in *N. vitripennis* are frequent enough for LMC theory to be relevant to our field populations. Given these data, *Nasonia* should have evolved as a result of LMC selection and be an ideal model organism to test assumptions of LMC. Table 2.3 gives an overview of the most important LMC model assumptions and the results of this study.

Table 2.3: Overview of assumptions made by several models on local mate competition theory and the results of this study

Reference	Assumption	Found in this study?
General assumptions	(1) Localized mating within patches	Yes
	(2) Random dispersal of mated females	Yes
Hamilton 1967	Equal clutch sizes	No
Hamilton 1967	Random offspring mating within patches	No
Werren 1980, Hamilton 1967, Frank 1985	Synchronous parasitism	No
Nunney and Luck 1988, Shuker <i>et al.</i> 2005	Asynchronous parasitism	Yes

Fragmented populations?

A general assumption in LMC theory is that the population is highly subdivided in terms of mating. This is usually thought to be a consequence of the patchy distribution of hosts. Our data confirm that patches are often parasitized by only one female, leading to very localized mating. LMC theory then assumes that mated females disperse randomly from their natal patch. Consistent with this,

the individual based test for isolation by distance did not show an increase of genetic differentiation with geographic distance within localities (Figure 2.4). This lack of differentiation between patches is also shown by the equal level of genetic distance within and between patches of one locality. Using the conventional F-statistic as developed by Weir & Cockerham (1984) to compare the localities, we find a rather low degree of differentiation between the two sampling localities ($F_{LT} = 0.035$). The private allele method estimates the number of migrants per generation between the populations and can be interpreted as a F_{LT} of 0.11. This resembles more considerable differentiation and is in the same range as Hedrick's G'_{LT} of 0.23. Together, these data indicate that there is high dispersal within the scale of the localities and that the composition of foundresses parasitizing a patch represents a random genetic sample of the local population. Therefore the relatedness among the foundresses of a patch can be expected to be similar to that within a locality. Between the two localities (HV and Schl) however, gene flow seems to be very limited, as expected by the large distance of about 300 km. The low differentiation indicated by the conventional F-statistics can easily be explained by the high variation of the used markers (Hedrick 2005). Therefore, the variation independent measurements G'_{LT} and the private allele method should be more informative. This leads to the conclusion that the relevant scale for LMC is the hierarchical level of localities, and not the total sample.

A common measure of the level of relatedness in a population is the population inbreeding coefficient F_{IT} . Hamilton (1979) predicted that, under the assumptions of random mating within a patch and equal foundress productivity, the population inbreeding under LMC should follow $F_{IT} = 1/(4n-3)$, with n being the harmonic mean number of foundresses per patch. For our study n is 1.9, resulting in an expected population inbreeding coefficient of 0.22 which is very close to the observed value of $F_{IL} = 0.168 \pm 0.016$. We use F_{IL} rather than F_{IT} , as the relevant level for LMC is the local population rather than the total sample as discussed above. However, the assumption of equal productivity is clearly violated and mating within a patch might not be random (as a consequence of asynchronous parasitism; see below). Therefore Hamilton's prediction can only be seen as a rough estimate.

Molbo and Parker (1996) calculated a population inbreeding coefficient F_{IT} of 0.312 for a Swedish population, which is considerably higher than our study. However, Molbo and Parker used all genotyped individuals for a calculation of F_{IT} , in contrast to our study (Molbo, personal communication). A recalculation of F_{IT} in our study using all individuals results in 0.272 ± 0.042 which more closely resembles the value of Molbo and Parker (1996). Moreover as Molbo and Parker (1996) used allozymes, the probability of underestimating the real number of foundresses due to limited variation in the marker is much higher than with the microsatellites we used ($\sim 10\%$ Molbo and Parker 1996, $< 1\%$ this study). In addition they estimated 1.5 foundresses per patch, while our estimate is 1.9. We also found a higher level of superparasitism (41%) than Molbo and Parker (23%). These differences could be explained by the higher resolution of our microsatellite markers, or by ecological differences between their Swedish population and our Dutch and German populations (such as population densities of parasites and hosts). An overall inbreeding coefficient F_{IL} of 0.168 corresponds to 45% sibmating (using $S = 4F_{IT}/(1+3F_{IT})$, Werren 1987). This is in the same range as the proportion of sibmating that has been found for *Trichogramma pretiosum* (56.6%, Antolin 1999), a gregarious parasitoid of Lepidoptera.

Equal clutch sizes and random mating within patches?

Hamilton (1967) assumed in his original LMC model that there is random mating among all the offspring on a patch and that all females in a patch lay equally sized clutches. Unsurprisingly, females lay varying clutch sizes, and there is a large coefficient of variation in clutch sizes per pupa across the patches (0.69 ± 0.04). This variation could be a consequence of sequential parasitism where the first female usually lays the largest clutch and later females lay reduced clutches (Werren 1980).

Unfortunately we cannot measure deviations from random mating on patch level using our data. One way to do that would be to measure the relatedness between foundresses and their mates. As *Nasonia* males are haploid and we have only information from four microsatellite loci, such measurements would be rather limited in this context, and we therefore did not present such analysis here. However, we can draw some conclusions from our other findings. The data strongly suggest that parasitism of hosts on a patch is asynchronous (see

next section for details), which leads to a bias in the opportunities for individuals from different hosts to mate with each other, as the daughters of early foundresses might have already left when the sons of late foundresses emerge. The sons of early foundresses on the other hand will have the chance to mate with their early sisters as well as with the daughters of late foundresses, as they stay on the patch. This obviously leads to the conclusion that mating among the offspring of a patch cannot be completely random, but only among the offspring that are present at the same time. (Shuker *et al.* 2006a).

Synchronous parasitism?

If all foundresses parasitized hosts at the same time, one would expect no increase in the emergence window with foundress number. As the emergence window on patch and host level is strongly positively correlated with foundress number (Figure 2.2), synchronous parasitism is perhaps the exception rather than the rule (Werren 1980, Hamilton 1967, Frank 1985). However, alternative explanations for the emergence window exist, including delayed developmental time due to crowding in the hosts, or individual foundresses parasitizing the same host several times, which might occur given the large variation in emergence time of the offspring of single foundresses (Figure 2.2B). Multiple parasitism by a single foundress on the same host may change the optimal sex ratio towards more males, if the female parasitized other hosts in between, as found by King (1992). Werren (1980) found that asynchronously laid clutches are synchronized by a speed up of development of the later clutches. Such a behavior would lead to a weaker correlation between foundress number and emergence window, than is evident from our data. However, we only collected data on the emergence window per host. To be able to resolve parasitism strategies of individual foundresses we would need data on the emergence time of individual offspring. Nevertheless, LMC models for species such as *Nasonia vitripennis* should incorporate asynchronous parasitism, as is the case in some more recent models (Nunney & Luck 1988; Shuker *et al.* 2005).

Additional parameters

In addition to the assumptions from existing LMC theory (Table 2.3) that were tested, we also considered some other parameters. Although the total number of hosts may intuitively be considered as a good predictor of patch quality, we did

not find a significant correlation between foundress number and the total number of hosts in a patch. One reason for this might be variation in individual host quality across patches. Also, variation in age of the hosts might play a role in the attractiveness of a patch. Hosts can only be parasitized by *N. vitripennis* if they are at a certain stage of development. If a patch has a large number of hosts suitable for parasitism for a longer period of time due to variation in host age, it might attract more wasps than a patch with an equal number of hosts that are all the same age. This would also explain the inferred patterns of sequential oviposition.

As superparasitism constitutes direct resource competition for a particular host, one may expect that the foundresses have evolved ways to avoid each other when parasitizing the same patch, as has already been shown in several studies (e.g. Shuker *et al.* 2005). Such a behaviour would lead to an underdispersed pattern of parasitism. However, our results do not indicate a significant deviation from a random pattern of parasitism. We should mention though that our sample sizes, especially on patch level, are rather low and that the goodness of fit test that was applicable for our data is not very powerful. Hence, at patch level, we have no strong evidence for preference or avoidance of superparasitism.

The estimated percentage of unmated females (2%) is in the range of what has previously been reported: Beukeboom and Werren (2000) found $2.99\% \pm 2.32\%$ in a larger field sample from the US. This frequency of so-called constrained females should not have a strong effect on the expected optimal sex ratio at the level of the population (Godfray 1990; Hardy & Godfray 1990). We assumed that the all-male family in a one-foundress patch in our study was due to an unmated female. We also found some all-male families among superparasitized hosts. In these latter cases family sizes were small and the assigned female also produced daughters in other hosts. Hence, such small all-male families can be considered as the outcome of superparasitism as predicted by LMC (Werren 1984).

Although previous studies indicated that single mating appears to be the rule in *Nasonia* (Azab *et al.* 1967; van den Assem & Visser 1976; van den Assem 1977), we found evidence that a small proportion (2 out of 49, $\sim 4\%$) of foundresses are multiply mated. Genotyping errors can almost be ruled out, as

we genotyped doubtful individuals at least twice, but the high level of inbreeding indicates that there is a high chance of highly related foundresses that have similar genotypes. If there would be the tendency that highly related females parasitize the same patch, there should be a correlation between genetic and geographical distance on a local scale. Our isolation by distance analysis however did not show any indication of such a correlation (Figure 2.4).

In general, polyandry reduces relatedness among the female offspring of a particular female. Unlike inbreeding, which would lead to selection for a more female biased sex ratio (Reece *et al.* 2004; Shuker *et al.* 2004a), polyandry does not change the relatedness of a mother to her offspring and should therefore have no influence on sex allocation. It has been shown that multiple mating in *N. vitripennis* increases with time cultured in the lab (van den Assem & Jachmann 1999; Burton-Chellew *et al.* 2007a). Furthermore, van den Assem and Visser (1976) showed that females are willing to mate a second time when they have already laid eggs. Therefore, it is conceivable that a previously mated female encounters a male that was born on the patch where she is ovipositing and mates a second time outside her natal patch. Nevertheless multiple mating seems to be rare in *N. vitripennis* and the effect of this behavior on the population genetic structure is likely to be negligible.

Finally, as predicted, we found a strong positive correlation between sex ratio and number of foundresses per patch, although there were large quantitative deviations from the predictions of Hamilton (1967) and Frank (1985b). We consider sex allocation in more detail elsewhere (Burton-Chellew *et al.* 2008).

To summarize our findings we can state that a suitable model of LMC for species such as *Nasonia vitripennis* should make the following assumptions: (1) large variation in clutch sizes, (2) non-random mating within the offspring of a patch, (3) asynchronous parasitism, (4) regular encountering of competitors, (5) highly structured mating populations (within localities) followed by (6) a random distribution of foundresses across the patches, and across hosts within patches. More recent models of LMC have started to take such factors into account (Nunney & Luck 1988; Shuker *et al.* 2005). Our findings provide empiric values for these factors and this will help to develop more realistic and precise LMC models, and hopefully also stimulate much needed studies of sex allocation in the wild for a wider range of parasitoid species.

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